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## Effects of Mutations in the Polymerase Domain on the Polymerase, RNase H and Strand Transfer Activities of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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Based on structural analyses and on the behavior of mutants, we suggest that the polymerase domain of HIV-1 reverse transcriptase (RT) plays a critical role in holding and appropriately positioning the template-primer both at the polymerase active site and at the RNase H active site. For RT to successfully copy the viral RNA genome, RNase H must cleave the RNA with absolute precision. We believe that a combination of the structure of the template-primer and its precise positioning are responsible for the specific cleavages RNase H makes. We have proposed that resistance of HIV-1 RT to nucleoside analogs involves a subtle repositioning of the template-primer. This hypothesis is based on both structural and biochemical analyses. Mutations that confer resistance to nucleoside analogs do not cluster at the polymerase active site; however, they are in positions where they could alter the interaction between RT and the template-primer.

If, as we have hypothesized, the polymerase domain is primarily responsible for positioning the template-primer and RNase H cleavage depends on this positioning, it should be possible to use RNase H cleavage to monitor at least some of the major changes in the position of the template-primer. We have used three assays (polymerase, RNase H, and strand transfer) to investigate the effects of mutations in the polymerase domain, including mutations that confer resistance to nucleotide analogs, on HIV-1 RT. All three assays involve RNA sequences derived from the viral genome. The data show that alterations in the polymerase domain, in particular, mutations that are in positions that would be expected to alter the interaction of RT with the template-primer, can alter both the efficiency and specificity of RNase H cleavage. These results are discussed in light of the structure of HIV-1 RT.

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### Introduction

Retroviral reverse transcriptases (RTs) contain both a DNA polymerase, which can copy either an RNA or a DNA template, and a ribonuclease H (RNase H), which cleaves RNA only if it is in RNA-DNA hybrid. These two activities cooperate

to convert the single-stranded RNA genome found in virions into double-stranded DNA (Weiss *et al.*, 1982, 1985; Varmus, 1988; Goff, 1990; Whitcomb & Hughes, 1992).

The RT of human immunodeficiency virus type-1 (HIV-1) is a heterodimer composed of a p66 subunit and a p51 subunit. The p66 subunit, which is 560 amino acid residues in length, contains both the polymerase and RNase H domains. The amino-terminal portion of HIV-1 RT forms the polymerase domain; the C terminus forms the RNase H domain. Proteolytic cleavage of the p66 subunit (or

Abbreviations used: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; ds, double-stranded.

some larger Gag-Pol precursor) at position 440 yields the p51 subunit, which corresponds closely to the polymerase domain of the p66 subunit (Di Marzo Veronese *et al.*, 1986; Larder *et al.*, 1987; Hansen *et al.*, 1988; Hizi *et al.*, 1988; Hughes *et al.*, 1998; Hostomsky *et al.*, 1991; Howard *et al.*, 1991; Skalka & Goff, 1993). The crystal structure of HIV-1 RT (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993) has been likened to a human right hand and based on this similarity; the polymerase domain has been divided into fingers, palm, thumb, and connection subdomains (Kohlstaedt *et al.*, 1992). While the folding of these four individual subdomains is similar in the two subunits, the relative positions of the subdomains in the two subunits are quite different. As a consequence, p66 is an extended structure with a large cleft in which double-stranded nucleic acids can bind, while p51 is more compact (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993; Nanni *et al.*, 1993).

A triad of aspartic acid residues (positions 110, 185, and 186) in the palm subdomain of the p66 subunit forms the polymerase active site. Like many other DNA polymerases, HIV-1 RT requires both a primer and a template strand. The double-stranded portion of the template-primer lies in the cleft formed by the fingers, palm, and thumb of p66; elements of this portion of p66 appear to function as a clamp that positions the template-primer relative to the polymerase site (Jacobo-Molina *et al.*, 1993; Boyer *et al.*, 1994a). We believe that the proper positioning of the template-primer is critical for both the polymerase activity and RNase H activity.

HIV-1 RT has an essential role in the viral replication and is an important target for antiviral therapy. To design better anti-RT drugs, we need to understand the structure and function of HIV-1 RT and to understand how mutations in HIV-1 RT engender drug resistance. A real understanding of this problem will require an exploration of the drug-resistant variants now known, and better understanding of the limits of the genetic and physical flexibility of HIV-1 RT. Because of the importance of the interactions of HIV-1 RT with the template-primer, we are particularly interested in mutations that affect the proper positioning of the template-primer.

Here, we have focused on three classes of mutations, all of which we believe are involved in nucleic acid positioning. The first class consists of mutations in the polymerase domain (either fingers or palm) that were previously shown to preferentially affect RNase H activity, and to have a more modest effect on polymerase activity. Since these mutations retain relatively high levels of polymerase activity, the defect in RNase H activity would be the result of template-primer repositioning, and not simply a general effect on nucleic acid binding.

We have previously suggested that at least some of the mutations that engender resistance to nucleoside analogs also involve a repositioning of the template-primer (Boyer *et al.*, 1994a). Since these mutations can be readily selected by chal-

lenge with a drug, the effects on the ability of RT to copy the HIV-1 genome, and by extension, on the polymerase and RNase H activities of the RT, are likely to be relatively minor. However, we wanted to test this class of mutants to see if, using appropriately sensitive assays, we could detect any subtle, but measurable, effects.

The third class of mutations are in the thumb. We chose to study mutations in the thumb primarily because the structure of HIV-1 RT suggests that the thumb, which has significant contacts with the template-primer, might play an important role in determining the position of the template-primer.

To determine the effects of individual amino acid substitutions on the polymerase and the RNase H activities, a series of mutants was generated in the fingers and palm (Figure 1(a)) and in the thumb of HIV-1 RT (Figure 1(b)) and analyzed using quantitative *in vitro* polymerase, RNase H cleavage and strand transfer assays similar to those described (Huber *et al.*, 1989; Krug & Berger, 1989; Hu & Temin, 1990; Luo & Taylor, 1990; Schatzet *et al.*, 1990; Furfine & Reardon, 1991; Peliska & Benkovic, 1992; Boyer *et al.*, 1994c).

The strand transfer assays were done to see if any of the mutants might affect some sort of specialized strand transfer activity or whether, in these assays, strand transfer would appear to be a simple combination of the polymerase and RNase H activities.

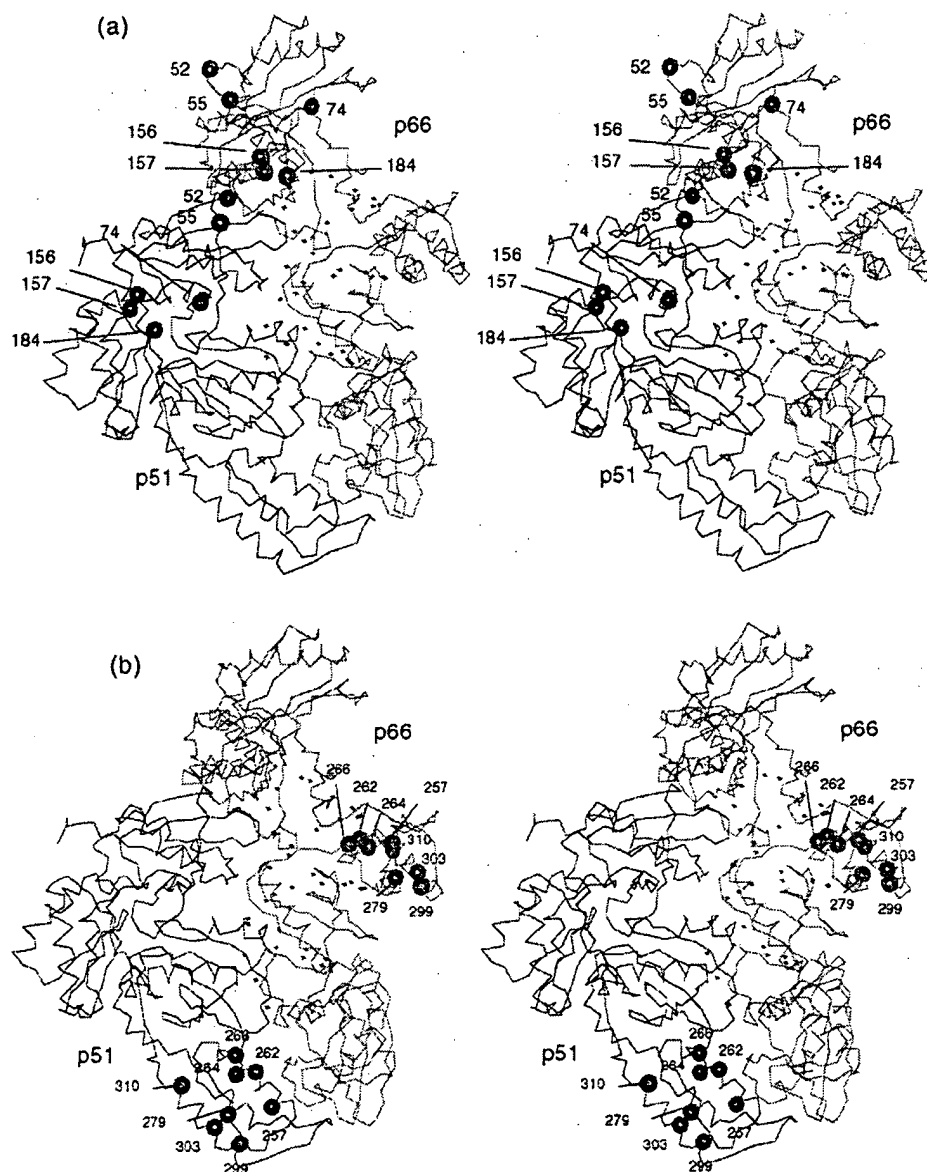
## Results and Discussion

### RNA-dependent DNA polymerase activities of HIV-1 RT mutants

A series of HIV-1 RT mutants was prepared that have amino acid substitutions in the fingers, palm, to thumb subdomains. Some of these mutants had been assayed for DNA dependent and RNA dependent polymerase activity (Boyer *et al.*, 1992a,b); all of the mutants were assayed to determine their ability to copy sequences in a 198 base RNA template (see Figure 6(a)) using a 20 bp (<sup>32</sup>P)-labeled DNA primer (see Materials and Methods). The RNA template in the assay is derived from the HIV-1 genome. The DNA products were fractionated on a denaturing 6% polyacrylamide gel. The gels were analyzed both by autoradiography and phosphorimager analyses (Table 1). Most of the mutants were less efficient in copying the template than HIV-1 RT. It was apparent, when compared with wild-type RT that all the mutants had a greater tendency to pause than wild-type RT (Figure 2 and data not shown). However, a careful look at the pausing patterns of the individual RTs showed that the pause sites that were most affected differed from one mutant to another. Computer analyses have suggested that the RNA genome of HIV-1 has a complex secondary structure. The RNA template used in the assay was analyzed using an RNA secondary structure program (DNA-SIS Hitachi Software). This analysis predicts that

there are several hairpin structures in the RNA used as a template, one of which corresponds to TAR. A comparison of the predicted secondary structure and the pattern of pause sites on the

RNA template suggests that the enzyme pauses preferentially at the predicted hairpins (including TAR) and that the mutant RTs are more susceptible to pausing at secondary structure elements



**Figure 1.** Stereodiagram of the C $\alpha$  backbone of the HIV-1 RT/dsDNA complex depicting the location of the mutations in the crystal structure. (a) The C $\alpha$  backbone of the HIV-1 RT p66/p51 heterodimer and the phosphate backbone of the template-primer duplex derived from the 3.0 Å resolution structure of an HIV-1 RT/dsDNA complex (Jacobo-Molina *et al.*, 1993). The locations of the mutations in the fingers and palm subdomains are shown in both the p66 and p51 subunits. The Figure was generated using the molecular modeling program Insight II (Biosym/MSI, San Diego, CA). The C $\alpha$  backbone of the p66 subunit is in red, while the C $\alpha$  backbone of the p51 subunit is in blue. The phosphate groups in the sugar backbone that define the position of the double-stranded template-primer are shown as black crosses. The locations of the mutations are shown as black disks labeled with the corresponding amino acid numbers. Positions 52 and 55 are in the fingers subdomain (in the  $\beta$ 2 and  $\beta$ 3 loop) as is position 74 (in strand  $\beta$ 4). The palm subdomain contains positions 156, 157 (in strand  $\alpha$ E) and 184 (in the  $\beta$ 9 and  $\beta$ 10 loop), which is next to the polymerase active site, defined by the catalytic triad (positions 110, 185 and 186). The polymerase active site is in the palm subdomain of the p66 subunit. (b) the positions of the mutations in the thumb subdomain are shown using a similar structural diagram.

Table 1. Polymerase, RNaseH and strand transfer activities of HIV-1 RT mutants

HIV-1 RT mutant	Location <sup>a</sup>	Polymerase (%) <sup>b</sup>	RNase H (%)	Strand transfer (%)
P52G	F	26	14	2
P55G	F	54	43	5
S156A	P	26	44	3
S156G	P	0	96	0
S156T	P	0	95	0
S156A/P157G	P	1	50	1
P157G	P	66	98	46
L74V	F	103	105	266
M184I	P	46	101	85
M184L	P	10	98	2
M184V	P	52	102	74
I257T	T	4	55	0
G262A	T	0	75	0
L264S	T	83	70	20
W266T	T	0	13	0
L279S	T	79	94	32
A299L	T	32	90	0
L303S	T	41	92	0
L310S	T	59	98	20
E478Q	R	100	0	0

All assays were independently performed three times with similar results.

<sup>a</sup> F, fingers; P, palm; T, thumb; R = RNase H.

<sup>b</sup> Polymerase activity was determined according to the amount of full-length DNA product.

than is the wild-type enzyme (data not shown). In the Introduction, the mutants were divided into three classes. (1) Mutations in the fingers and palm that were believed, on the basis of previous work, to preferentially affect RNase H activity relative to polymerase activity. (2) Nucleoside-resistant mutants (believed to involve nucleic acid repositioning). (3) Mutations in the thumb.

The original analyses used to define the polymerase activity of the first mutant class involved the measurement of the incorporation of a radioactive dNTP using a homopolymeric template. In the experiments described in Table 1, we are using a more stringent test: measuring the ability of the RT to synthesize a product of some 139 bases (including the primer) from a viral RNA template that has substantial amounts of predicted secondary structure. Some of the mutants (P52G and S156A) make only about one quarter the amount of the 139 base product as does wild-type HIV-1 RT, while P55G makes about half as much of this product as wild-type RT and P157G about two-thirds as much. The double mutant S156A/P157G synthesizes only about 1% as much product as does wild-type RT.

Of the second class of mutants, the drug-resistant variant L74V has essentially the same ability to make the 139 base long product as wild-type HIV-1 RT; however, the variants M184I and M184V make only about half as much full-length product. The variant M184L, which shows resistance to ddITP in *in vitro* assays but is not selected by drugs, is significantly deficient in its ability to make the 139 base product (10% of wild-type). These results are similar to data that were obtained in a processivity assay using a DNA template (Boyer & Hughes, 1995); however,

smaller differences were seen in the processivity assay. Since the polymerase assay used here permits multiple rounds of synthesis, differences in processivity can be amplified.

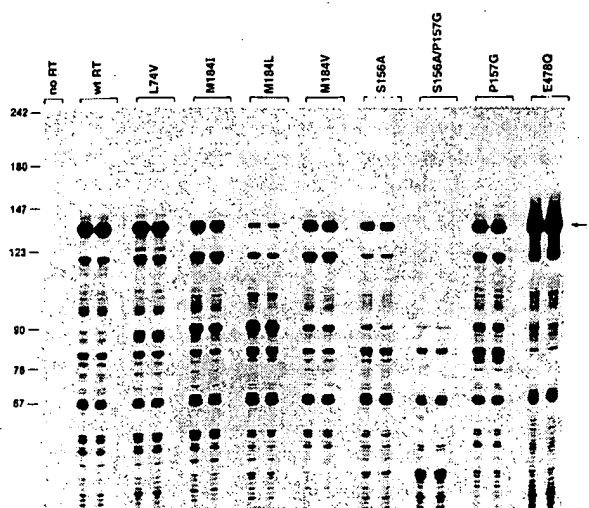
The third class of mutants is in the thumb. In HIV-1 RT, the thumb is composed of three  $\alpha$  helices ( $\alpha$ H,  $\alpha$ I and  $\alpha$ J).  $\alpha$ H makes contact with the primer strand,  $\alpha$ I with the template strand (Jacobo-Molina *et al.*, 1993). Of the mutants we examined, several of the mutations in  $\alpha$ H (I257T, G262A and W266T) had a profound effect on the ability of RT to make the 139 base product. In contrast, none of the other mutations we tested in the thumb, L264S, which is also in  $\alpha$ H, the mutation in  $\alpha$ I (L279S), and the three mutations in  $\alpha$ J (A299L, L303S, and L310S) had as great an impact on polymerase activity as any of the mutations in  $\alpha$ H.

In earlier studies, Beard *et al.* (1995, 1996) and Bebenek *et al.* (1995) measured the effects of alanine substitutions in both  $\alpha$ H and  $\alpha$ I. A direct comparison of their data and ours is difficult, since the mutations themselves, the form of the HIV-1 RT used in the experiments (homodimer *versus* heterodimer), and the assays used to measure polymerase activity are all different. However, the authors argued in their most recent paper (Beard *et al.*, 1996) that  $\alpha$ H plays a more important role than  $\alpha$ I in interacting with nucleic acid.

The RNase H negative mutant, E478Q, was included in all of the experiments as a control. This mutant RT synthesizes the same amount of the 139 base product as wild-type RT.

#### RNase H activity

Mutations in the polymerase domain can profoundly affect RNase H activity (Boyer *et al.*, 1992a,b, 1994c). There can be trivial explanations



**Figure 2.** Analysis of the RNA-dependent polymerase activities of wild-type and mutant HIV-1 RTs. A 198 nucleotide RNA template, consisting of the repeat (R) and unique 5' (U5) sequences in the 5' LTR of HIV-1 genome, was generated using linearized pBLR and T7 RNA polymerase (see Materials and Methods). A 5' end-labeled DNA primer, which is complementary to the sequences in the RNA template, was annealed to the RNA template. After addition of dNTPs,  $MgCl_2$ , and HIV-1 RT, the primer is elongated to generate the DNA product. After primer elongation, the reaction products were separated on a denaturing 6% polyacrylamide gel. The gel was dried and autoradiographed. All assays were performed in duplicate. The full-length DNA product (F) is 139 nucleosides long. The molecular mass markers are  $^{32}P$ -labeled, *MspI*-digested pBR322. Assays were done in duplicate and the duplicate assays analyzed in adjacent lanes.

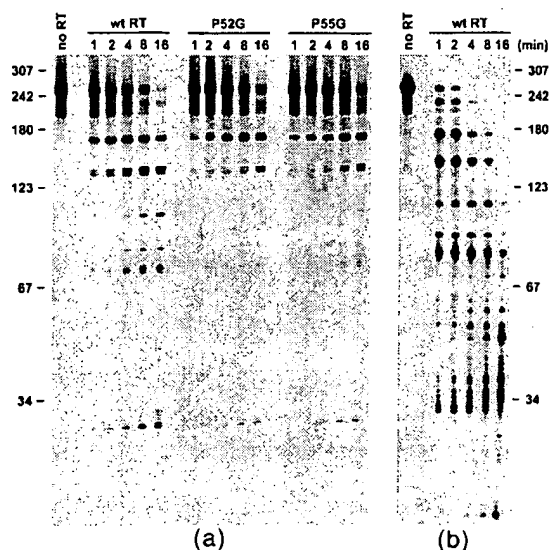
for such results: for example, some mutations could prevent RT from forming an active heterodimer or prevent RT from binding to the template-primer. However, such mutations would necessarily affect both polymerase and RNase H more or less equally. We are particularly interested in mutations in the polymerase domain that preferentially affect RNase H activity. An examination of the structure of HIV-1 RT suggests two possible mechanisms. The two possible explanations are not mutually exclusive: the effects of some mutations could involve one mechanism, other mutations the alternative; in some cases both mechanisms may be involved. The first mechanism depends on the fact that the p66 polymerase domain is primarily responsible for holding and appropriately positioning the nucleic acid (Jacobo-Molina *et al.*, 1993; Nanni *et al.*, 1993). Thus, RNase H cleavage (both efficiency and specificity) depends on appropriate positioning of the nucleic acid by the polymerase domain. An examination of the structure of HIV-1 RT in complex with nucleic acid (Jacobo-Molina *et al.*, 1993) shows that some of the mutations that

affect RNase H activity lie in parts of the polymerase domain of HIV-1 RT (fingers and palm) where the change in the p66 subunit would affect the positioning of the template-primer.

The second explanation is that a mutation in the polymerase domain, rather than affecting the positioning of the nucleic acid, affects the structure or position of RNase H itself. The portion of the polymerase domain that makes the most extensive contacts with RNase H is the thumb subdomain, in particular, the thumb of p51 (see Figure 1). The complication is that the thumb of p66 has close contacts with the nucleic acid; simple inspection of the structure does not make it possible to decide whether a mutation in the thumb subdomain affects the nucleic acid position, RNase H, or both. These complications require additional analyses; it is necessary to determine which subunit (p66 or p51) is responsible for the phenotype of a thumb mutant.

In order to quickly monitor the ability of RNase H to cleave a number of different sites in a single RNA, a group of seven oligonucleotide DNA primers were annealed to labeled RNA template. Under conditions where the wild-type enzyme degraded essentially all of the template RNA, the amount of cleavage produced by the class I mutants P52G, P55G, S156A and S156A/P157G was lower than wild-type RT. Of these, the most profoundly affected was P52G, which has only about 14% of the wild-type RNase H activity in this assay. In contrast, S156G, S156T and P157G all show normal levels of RNase H activity using this assay (Table 1).

We had previously studied the RNase H activity of these mutations using an *in situ* assay that measured the activity of p66/p66 homodimers that were renatured following SDS-PAGE. In the *in situ* renaturation assays the S156A and P55G mutants showed no measurable RNase H activity. We believe that these mutants were able to renature following electrophoresis since, in parallel experiments, these same mutants showed DNA-dependent polymerase activity following renaturation after SDS-PAGE (Boyer *et al.*, 1992a). One major difference in the experiments was that, in the renaturation experiments, the RNase H activity was measured using a p66/p66 homodimer form of HIV-1 RT; in the experiments we report here, RNase H activity is measured by using a p66/p51 heterodimer of HIV-1 RT. It should also be pointed out that the *in situ* RNase H assay is relatively insensitive, since it depends on there being enough activity to degrade all of the labeled RNA in the vicinity of the renatured enzyme, creating a small unlabeled band in an otherwise uniformly labeled gel. In contrast, the assay we have used here is quite sensitive; it can detect single cleavages anywhere along the template RNA, so it may not be surprising that two of the mutants that were scored as negative in the *in situ* RNase H assay have considerable activity with the defined template. The defined template assay is not only sensi-



**Figure 3.** RNase H activity of wild-type and mutant HIV-1 RTs. A 267-nucleotide RNA template containing the R, U5 and primer binding site sequence located at the 5' end of the HIV-1 genome was generated as described in Materials and Methods. The RNA template was labeled, during synthesis, with [ $\alpha$ - $^{32}$ P]UTP using T7 RNA polymerase. The RNA template was designed to contain a short poly(A) tract at its 3' end. Full-length RNA was purified from partial products using magnetic beads carrying oligo(dT) (see Materials and Methods). Labeled full-length RNA (\*) was hybridized to a set of seven complementary DNA oligonucleotides. Multiple oligonucleotides are used to test multiple cleavage sites on a single RNA template. (a) The RNase H cleavage reaction was initiated by the addition of 20 ng of purified mutant HIV-1 RT or 8 ng of purified wild-type RT in the presence of 5 mM MgCl<sub>2</sub> and was allowed to proceed for times that varied between 1 and 16 minutes at 37°C. The amount of wild-type HIV-1 RT was reduced to compensate for the lower activity of the mutants. The effects of digesting with 20 ng of wild-type HIV-1 RT is shown in (b). The reaction was stopped with 2 × RNA loading buffer and the products were separated on a denaturing 6% polyacrylamide gel. The gel was dried and autoradiographed. The molecular mass markers are  $^{32}$ P-labeled, *MspI*-digested pBR322.

tive, it is specific. As expected, the RNase H mutant E478Q shows no detectable activity in this assay.

The class 2 (nucleoside analog-resistant) mutants all showed normal levels of RNase H activity using the defined template assay, suggesting that the effects that these mutations have on template-primer positioning are not propagated all the way from the polymerase active site to the RNase H active site.

In contrast to the relatively profound effects of some of the mutations in the thumb (in particular mutations in  $\alpha$ H) on polymerase activity, the effect of these mutations on RNase H activity are, in general, much less dramatic. Only one of the mutants

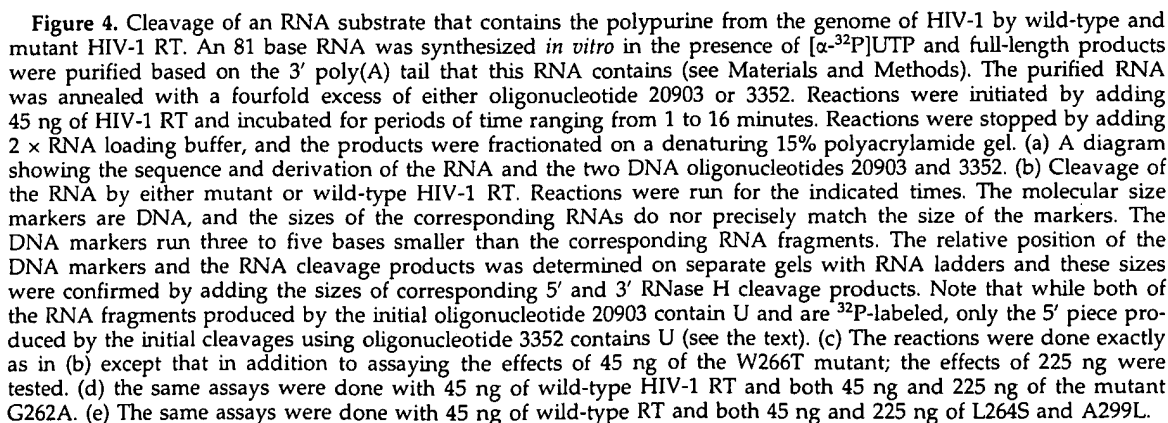
(W266T) had less than 50% of the RNase H activity of wild-type HIV-1 RT in the assay using the long RNA template and seven oligonucleotides.

We were particularly interested in the possibility that some of the mutations in the polymerase domain altered both the efficiency of RNase H cleavage and its specificity. A time-course analysis showed that if the time of digestion and the amount of enzyme was appropriately increased, the digestion pattern with the mutants P52G and P55G was quite similar to wild-type (Figure 3).

A number of laboratories have used the specificity of RNase H cleavage at the polypurine tract to study retroviral RTs (Luo & Taylor, 1990; Wörlh & Moelling, 1990; Randolph & Champoux, 1994; Guo *et al.*, 1995). This is a region where the specificity of RNase H cleavage is especially important and we prepared a second RNA template that contains the HIV-1 polypurine tract, together with 20 base complementary oligonucleotides. Two of these DNA oligonucleotides (20903 and 3352, see Figure 4), in complexes with the synthetic RNA, were particularly useful in discriminating between wild-type and mutant HIV-1 RTs.

Wild-type HIV-1 RT can cleave the RNA when it is complexed with either of the DNA oligonucleotides. However, cleavage was more efficient with oligonucleotide 3352. This is to be expected, since the oligonucleotide 20903 hybridizes directly to the polypurine tract and the expected site for the initial cleavages, approximately 17 to 18 bases from the 3' end of the oligonucleotide, is within the polypurine tract itself, in a sequence where cleavage is restricted during viral replication (Champoux, 1993; Coffin *et al.*, 1997; Hughes *et al.*, 1998). Under the conditions employed in our assay, with a relatively short DNA oligonucleotide, wild-type HIV-1 RT can cleave within the polypurine tract, approximately 31 to 35 nucleotides from the 5' end of the RNA, which is at the expected position 17 or 18 bases from the 3' end of the DNA oligonucleotide. Prolonged incubation appears to produce additional cleavages at positions 36 to 39, which would lie beyond the end of the oligonucleotide. It may be that the structure of this single-stranded RNA region is influenced both by the double-stranded segment that is immediately adjacent, and by interactions with the RNase H domain, which may act together to allow cleavage where it would not normally occur. Although this was not the expected result, the significance is probably more for understanding the interaction(s) of RT and nucleic acid than for the replication of the HIV-1 genome: there does not appear to be an equivalent structure (a relatively long RNA complexed with a short DNA oligonucleotide) involved in the replication of the HIV-1 genome.

So far as we can tell, there are no additional secondary cleavages at positions around eight nucleotides from the 3' end of the DNA oligonucleotide (Ghosh *et al.*, 1995). Such a cleavage, if it occurred, would be in the middle of the polypurine tract. We assume that the sequence of the polypurine tract



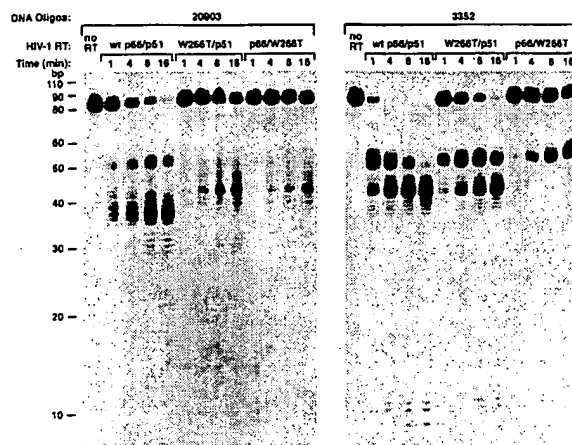
(or more accurately the structure, which depends on the sequence) does not permit secondary RNase H cleavages.

The complex of the RNA with oligonucleotide 3352 is cleaved essentially normally by wild-type HIV-1 RT, with the initial cleavages centering around positions that are approximately 17 or 18 bases from the 3' end of the DNA oligonucleotide (cleavage primarily at RNA positions 48 to 50), and secondary cleavages centering some seven or eight bases from the 3' end of the DNA oligonucleotide (cleavages primarily at RNA positions 39 to 42). The fact that the RNA substrate was labeled by incorporation of [ $\alpha$ - $^{32}$ P]UTP *in vitro* simplifies the digestion pattern produced with oligonucleotide 3352: all (or essentially all) of the labeled UTP is in the 5' portion of the RNA. Although the data are not as clear as the data with oligonucleotide 20903, a careful examination of the sizes of the bands does suggest that at least some cleavages can occur just beyond the end of the DNA oligonucleotide.

In contrast to wild-type HIV-1 RT, neither of the two mutants, W266T or G262A, is able to make significant cleavages within or near the polypurine tract using an RNA/DNA hybrid formed with the 20903 DNA oligonucleotide (Figure 4(a) and (d)). However, both can cleave the same RNA template using the 3352 oligonucleotide. The mutants G262A, L264S, and A299L, while considerably less active than wild-type HIV-1 RT with the 3352 oligonucleotide are able to cleave in the vicinity of -17 and -8 (Figure 4(d) and (e)). In contrast, W266T, which can make the -17 cleavages, does not make significant amounts of the -8 cleavages even when a fivefold excess of enzyme is used in the assay (Figure 4(c)).

As has been discussed, the positions of the thumbs in the structure of the HIV-1 RT are quite interesting: the thumb of p66 makes contact with the double-stranded nucleic acid between the polymerase and RNase H active sites, and is believed to play a crucial role in holding and positioning the nucleic acid. However, the thumb of p51 makes direct contact with the RNase H domain and, in addition, passes under the extended portion of the template-primer (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993). This means that alteration of either the thumb of p66 or the thumb of p51 could affect RNase H activity and specificity. We used subunit-selective mutagenesis to ask how the change(s) in the thumb of p66 or the thumb of p51 affected RNase H cleavage.

Neither the mutant with the W266T mutations only in p66, or only in p51, was able to cleave the 20903 complex efficiently, although both were more active than the enzyme with changes in both subunits. Moreover, the pattern of cleavage was different from the wild-type enzyme (see Figure 5). Although the digestion products produced by two subunit-selective mutants appeared similar with the 20903 oligonucleotide, there were significant differences in the cleavages seen with the 3352 oligonucleotide. The pattern of cleavage produced



**Figure 5.** Subunit selective effects of the mutation W266T on RNase H activity. The assay conditions were the same as those described for Figure 4 (see also Materials and Methods), except that the W266T mutation was present in the p66 subunit or in the p51 subunit.

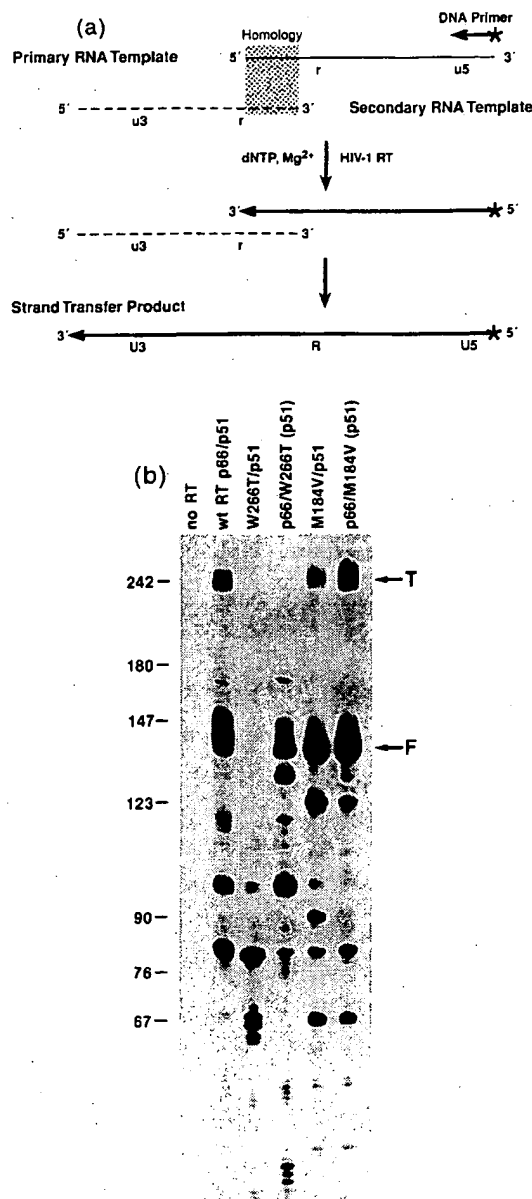
when the W266T mutation was in the p66 subunit was essentially normal (both the -17 and -8 cleavages are seen); however, when the W266T mutation is in the thumb of p51, only the -17 cleavages are seen, not the cleavages around -8.

These data show that both the thumb of p51 and the thumb of p66 are important for RNase H cleavage, which was also suggested by experiments in which the C280 position (which is in  $\alpha$ 1) was modified by *N*-ethylmaleimide (NEM). NEM modification of C280 selectively blocks RNase H activity without significantly altering polymerase activity. Subunit-selective mutagenesis showed that both the thumb of p51 and the thumb of p66 contributed to the sensitivity of the RNase H activity to NEM (Loya *et al.*, 1997).

Taken together, these results provide support for model in which the selectivity and specificity of RNase H cleavage depends on the structure of the RNA/DNA duplex, and on its interaction(s) with HIV-1 RT. Most of the interactions between nucleic acid and RT involve the polymerase domain, especially the polymerase domain of p66, and, as such, this domain plays a critical role in positioning the nucleic acid. The thumb of p66 has a direct role in this positioning, which appears to regulate the ability of RT to cleave in the vicinity of the polypurine tract. However, these results also point to a critical role for the thumb of p51, since mutations in the p51 thumb profoundly affect RNase H cleavage.

The precise role of the p51 thumb is still not entirely clear. Modification in the p51 thumb could affect the precise position of RNase H, the position of the template-primer, or both. Whichever model ultimately proves to be correct (and these two ideas are not mutually exclusive, so both may be





**Figure 6.** Analysis of subunit selective mutations on the DNA strand transfer activities of mutant HIV-1 RTs. (a) the same 198 nucleotide primary RNA template and oligonucleotide primer used in the polymerase assay were used in this assay. The acceptor (secondary) RNA template, which is 144 nucleotides long, is derived from U3 and R sequences. The R sequences are present also in the 5' sequence of the primary RNA template. In the strand transfer assay, the DNA primer was 5' end-labeled, then annealed to the primary RNA template. The acceptor RNA template was then added to the annealing reaction. Purified wild-type or mutant HIV-1 RT was added to this mixture in the presence of dNTPs and  $MgCl_2$ . The full-length transfer product is 238 nucleotides long. (b) The reaction was allowed to proceed for 60 minutes at 37°C and the products were then separated on a denaturing 6% polyacrylamide gel. The full-length transfer product is denoted by T and the full-length copy of the primary RNA is marked F. The molecular mass markers are  $^{32}P$ -labeled, *MspI*-digested pBR322.

valid), it is clear that the structure, and positioning of the nucleic acid substrate, relative to the RNase H domain, more particularly the RNase H active site, is of critical importance. In this regard, it is likely that it is the effect the polypurine tract has on the structure of the RNA/DNA duplex, rather than the sequence *per se*, that specifies the site(s) of RNase H cleavage.

### Strand transfer activity

Reverse transcriptase converts the single-stranded RNA genome of HIV-1 into double-stranded DNA. This process involves two transfers between templates, often referred to as the first and second jumps (Gilboa *et al.*, 1979; Varmus, *et al.*, 1979; Temin, 1981; Varmus, 1982; Telenitsky & Goff, 1993; Coffin *et al.*, 1997). The first jump involves the transfer of minus strand strong stop DNA from the 5' to the 3' end of the viral RNA genome. We have developed model RNA substrates that mimic the RNA segments involved in this first jump and have used these substrates to study the ability of the HIV-1 RT mutants to catalyze strand transfer *in vitro*.

The strand transfer reaction depends on both the polymerase and RNase H activities of RT. The requirement for polymerase is immediately obvious; RNase H is required to digest the RNA template strand so that the recently synthesized DNA strand can anneal to the second RNA template (see Figure 6(a)). It is appropriate, therefore, to link the ability of HIV-1 RT mutants to carry out strand transfer to their RNase H and polymerase activities. In particular, we are interested in whether the ability of HIV-1 RT mutants to carry out the strand transfer reactions correlates in some simple way with polymerase and RNase H activities. A simple relationship would imply that strand transfer does not involve any special or additional properties of HIV-1 RT; a more complex answer would suggest that strand transfer may require special properties of the enzyme beyond the polymerase and RNase H activities (Peliska & Benkovic, 1992). For some mutants (S156G, S156T and S156A/P157G, I257T, G262A and W266T), the polymerase activity is sufficiently deficient that synthesis does not reach the end of the first RNA strand (see Table 1). Such mutants are not able to carry out strand transfer.

Mutants lacking RNase H activity (for example the control enzyme E478Q, which completely lacks RNase H activity) are able to copy the first template efficiently; however, the inability of this enzyme to degrade the first RNA template leads to a complete failure in the strand transfer assay (Table 1). Several mutants that have relatively low RNase H activity (P52G for example) carry out little or no measurable strand transfer (Table 1). This is most simply explained based on the fact that, for strand transfer to occur, most or all of the RNA template must be removed from the DNA primer strand that is transferred in the strand transfer

reaction. As has already been discussed, the first cleavages made by RNase H center around position 17 or 18 bases from the 3' end of DNA primer, longer incubation leads to additional cleavages closer to the 3' end of the primer (near -8: Schatz *et al.*, 1990; Fu & Taylor, 1992; Gopalakrishnan *et al.*, 1992; Peliska & Benkovic, 1992; Cirino *et al.*, 1995; Ghosh *et al.*, 1995; Gotte *et al.*, 1995). Presumably these additional cleavages are necessary to free the DNA for the transfer. It is likely that the RNase H-deficient mutants that are unable to carry out strand transfer do not degrade the RNA sufficiently to free the end of the DNA for the transfer.

Subunit-selective mutagenesis with the W266T mutant showed that both the p66 and the p51 thumbs play important roles in RNase H activity. For this reason we also used the W266T mutant to test the roles of the p66 and p51 thumbs in polymerase activity and in strand transfer activity. These assays showed that when the mutation W266T is present only in the thumb of p66, it causes a major defect in polymerase activity. Since the DNA product does not reach the end of the first RNA template, there is no strand transfer (Figure 6(b)).

However, when the W266T mutation is present only in p51, there is considerable DNA product that represents a complete copy of the first RNA template, but no detectable strand transfer (Figure 6(b)). This is almost certainly due to the deficiency in RNase H cleavage. Since this subunit-selective mutant is deficient in both the total amount of RNase H activity, and in the ability to make the secondary cleavages around -8, we are not certain what leads to the failure of strand transfer. However, it does seem likely that the additional cleavages near -8 would be important in producing a product that would be used efficiently in a strand transfer reaction, and we would expect that mutations that would selectively impair secondary cleavages would be deficient in a strand transfer assay.

We prepared subunit-selective mutants of M184V to test which subunit is responsible for drug resistance (Boyer & Hughes, 1996). These subunit-selective mutants were included in the strand transfer experiments to see whether differences in strand transfer efficiency could be detected with this assay. The previous experiments showed that the drug resistance engendered by the M184V mutation acts through p66. The strand transfer experiments (Figure 6(b)) show that a change from M to V at position 184 in the p66 subunit increases the tendency of the enzyme to pause near positions 90 and 123. Making the corresponding M to V change at position 184 in p51 has no obvious effect in this assay. These results reinforce the idea (Coffin, 1995) that, in a highly adapted virus like HIV-1, mutations, including mutations that engender drug resistance, have some deleterious effect on the virus, and that the phenotypic effects of altering position 184 are the result of the changes in p66.

There are mutants whose strand transfer efficiencies are difficult to explain as alterations in the absolute level of either the DNA polymerase or RNase H activity. L74V, which appears to be relatively normal in both polymerase activity and RNase H activity, make more than twice as much full-length product in the strand transfer assay than the wild-type enzyme. This suggests the possibility that the p66 fingers, and, more specifically, the interaction of the p66 fingers with the template, may play an important role in the strand transfer reaction.

At the moment, with the data now available, we cannot explain the results obtained in the strand transfer assay with several of the mutants (for example, P55G, L264S, L279S, A299L, L303S) as defects in the absolute level of RNase H activity or the polymerase activity; however, it is possible that the defects in strand transfer seen with one or more of these mutants, will be due to an effect on the secondary RNase H cleavages near -8 rather than the absolute level of RNase H cleavage.

## Materials and Methods

### Preparation of p66/p51 heterodimers

*Bsp*MI cassette mutagenesis (Boyer *et al.*, 1992a) was used to construct HIV-1 RT mutants containing defined amino acid substitutions (Boyer *et al.*, 1994a; Boyer & Hughes, 1995). The cassette mutagenesis plasmids were derived from the plasmid RT(66) (Hizi *et al.*, 1988), which expresses the 66 kDa RT in *Escherichia coli*. The regions encoding these mutant RTs were modified so that codons for six histidine residues were inserted in the 3' end of the RT open reading frame just before the termination codon. RT open reading frames encoding either wild-type or mutant RTs were then cloned into a plasmid similar to the plasmid p6HRT-PROT (Le Grice & Grüninger-Leitch, 1990; Boyer *et al.*, 1992a, 1994b). The plasmids are based on the expression vector pT5m, and were introduced in to the *E. coli* strain BL21 (Studier & Moffatt, 1986; Rosenberg *et al.*, 1987; Le Grice & Grüninger-Leitch, 1990; Boyer *et al.*, 1994b). After induction with isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG), these plasmids express both the p66 form of HIV-1 RT (either wild-type or a mutant) and HIV-1 protease. Approximately 50% of the over-expressed p66 RT is converted to the p51 form by the HIV-1 protease, and p66/p51 heterodimers accumulate in *E. coli*. For some of the experiments, it was important to introduce the mutation of interest selectively into only one of the two subunits of HIV-1 RT. We have described plasmids that separately encode p51 and p66. These plasmids can be used to induce the synthesis of HIV-1 RT with specific amino acid substitutions either in p51 or p66 (Boyer *et al.*, 1994b; Boyer & Hughes, 1996). The p66/p51 heterodimers were purified by metal chelate chromatography (Le Grice & Grüninger-Leitch, 1990; Le Grice *et al.*, 1991; Boyer *et al.*, 1994a).

### Construction of plasmids used to synthesize RNA *in vitro*

A portion of the 5' LTR (from positions 455 to 660) of the HIV-1 provirus clone pNL 4.3 (Adachi *et al.*, 1986),

including the complete repeat (R) region, unique 5' (U5), and primer binding site, a total of 206 bp was cloned into the plasmid Bluescript SK (Stratagene, La Jolla, CA) at the *KpnI* and *XhoI* restriction sites using PCR and primers containing these sites. The resulting plasmid was designated as pBLR. A second plasmid was constructed from the same HIV-1 clone. A portion of the 3' LTR including unique 3' (U3) sequences (9117 to 9194) and a small portion of the vector and sequences from the 5' end of the insert in pBLR was cloned into the *EcoRI* and *HindIII* restriction sites of pGEM-3Zf(+) (Promega, Madison, WI). The resulting plasmid, pG89, has an insert of 140 bp. Of these 140 bp, the 46 bp at the 3' end of the insert are identical with sequences at the 5' end of the insert in pBLR. To simplify purification of RNA made *in vitro*, 30 adenine residues were attached to the end of the insert between the *XhoI* and *XbaI* sites of pBLR to create pBLRA30. All the plasmids inserts were verified both by restriction enzyme mapping and double-strand DNA sequencing.

For some of the RNase H assays, a plasmid was prepared that can be used to synthesize an RNA that contains the polypurine tract from the genome of HIV-1. This plasmid contains a segment that has 35 bases from the HIV-1 genome (positions 9049 to 9083) linked to 30 adenine residues, which was inserted into the *EcoRI* and *HindIII* restriction sites of the plasmid (pGEM-3Zf). The resulting plasmid, pGPA35, was also verified both by restriction enzyme mapping and double-strand DNA sequencing.

### RNA template preparation

The RNA templates used in the polymerase and strand transfer assays were synthesized *in vitro* from linearized plasmids (pBLR or pG89) by run-off transcription with phage T7 RNA polymerase using an RNA synthesis kit (MEGAscripts from Ambion, Inc., Austin, TX). The transcription reaction was heat inactivated at 70°C of 20 minutes and the RNA was subsequently purified by spin column chromatography using S-200-HR Sephacryl obtained from Sigma-Aldrich (St. Louis, MO). RNA concentration was determined spectrophotometrically. The RNA templates used in the RNase H cleavage assays were prepared from linearized plasmid DNA (pBLRA30 or pGPA35) by *in vitro* transcription in the presence of 120  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP. The resulting RNA was purified using the PolyAtract mRNA Isolation System composed of biotinylated oligonucleotide dT and streptavidin-coated magnetic particles (obtained from Promega, Madison, WI). The amount of radioactive UTP incorporation into RNA was determined by using a liquid scintillation counter.

### Polymerase assay

A 20 bp DNA primer (5'CACACACAACAGACGGGCACAC3', which corresponds to positions 122 to 103 in the HIV-1 genome), which is complementary to the 5' end of the U5 sequences of the RNA template, was 5' labeled with [ $\gamma$ -<sup>32</sup>P]ATP using phage T4 polynucleotide kinase according to New England Biolab's protocol. The labeled primer was mixed with the RNA template at a 4:1 molar ratio (primer/template) in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM KCl. The mixture was heated to 70°C for ten minutes and slowly cooled to room temperature. The RNA-dependent DNA polymerase reaction was initiated by adding

1  $\mu$ g of purified HIV-1 wild-type or mutant RT in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 6 mM MgCl<sub>2</sub>, 1 mM DTT, 100  $\mu$ g/ml acetylated bovine serum albumin, 10 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate), and 80  $\mu$ M each of dNTPs in a final volume of 10  $\mu$ l. Reactions were incubated at 37°C for 60 minutes and terminated by adding 2  $\times$  RNA loading buffer (85% (v/v) formamide, 50 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol). The products were heat-denatured and then separated on a denaturing 6% polyacrylamide/7 M urea gel in Tris-borate/EDTA buffer at 1600 V for approximately 90 minutes. The gel was dried and autoradiographed overnight, and the X-ray film was developed with a Kodak RP X-OMAT processor. The gel was also analyzed with a Molecular Dynamics Phosphorimager SF. The amount of the full-length DNA product was quantified by integration of volumes and subtraction of local background.

### RNase H cleavage assays

( $\alpha$ -<sup>32</sup>P)-labeled RNA template (50,000 cpm) synthesized from linearized pBLRA30 (~100 ng) was mixed with approximately 40 ng of seven oligonucleotide primers in the presence of 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2.0 mM DTT, 100  $\mu$ g/ml acetylated bovine serum albumin, 10 mM CHAPS. The RNAs synthesized from linearized pGPA35 containing the polypurine tract were hybridized to one of the two oligonucleotides 20903 (5'CCCCCTTTTCTTTAAAAA3', positions 8613 to 8632 in the HIV-1 genome) or 3352 (5'TTGTCGACCTTCCAGTCCC3', this oligonucleotide begins at position 8611 and ends in vector sequence; see Figure 4) under the same conditions. The mixtures of RNA and oligonucleotides were heated to 70°C for ten minutes and then slowly cooled to room temperature. For the seven oligonucleotide RNase H assay, reactions were initiated by adding 10 ng of purified wild-type or mutant HIV-1 RT and 60 mM MgCl<sub>2</sub> to a final concentration of 5 mM in a final volume of 12  $\mu$ l, and incubated at 37°C for four minutes or in a time-course as indicated for the Figures. For the assays done with RNAs containing the polypurine tract, 45 ng of wild-type or mutant HIV-1 RT was used. The reactions were terminated by adding 2  $\times$  RNA loading buffer. The products were heat-denatured and separated on either a 6% or a 15% polyacrylamide/7 M urea gel in TBE buffer at 1600 V for approximately 90 minutes. The gels were dried and autoradiographed overnight and were analyzed with a Molecular Dynamics Phosphorimager SF. The amount of the full-length RNA template remaining quantified by integration of volumes and subtraction of local background.

### Strand transfer assay

The same 20 bp oligonucleotide primer (5'CACACAACAGACGGGCACAC3') and the same primary RNA template used in the polymerase assay were labeled and annealed as described above. The secondary RNA template synthesized from linearized pG89 was then mixed with the hybrid in a molar ratio of 4:1. The strand transfer reaction was initiated by adding 1  $\mu$ g of purified wild-type or mutant HIV-1 RT in 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 6 mM MgCl<sub>2</sub>, 1 mM DTT, 100  $\mu$ g/ml acetylated bovine serum albumin, 10 mM CHAPS, and 80  $\mu$ M each of dNTPs in a final volume of 15  $\mu$ l. Reactions were incubated at 37°C for 60 minutes

and terminated by adding 2 × RNA loading buffer, and the products heat-denatured and then separated on a denaturing 6% polyacrylamide/7 M urea gel. Gels were run and analyzed as described above.

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